VirD2 of Agrobacterium tumefaciens : functional domains and biotechnological applications

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Summary

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Agrobacterium tumefaciens is a Gram-negative bacterium that naturally occurs in soil. In dicotyledonous plants, it causes the crown gall disease. *Agrobacterium* causes this disease by transferring a strand of DNA (the transferred or T-strand) of its tumor-inducing plasmid (pTi) into the plant cells. Once inside the plant cell, the T-strand enters the nucleus where it integrates into the genome of the plant. The genes encoded on the T-strand cause rapid plant cell division, resulting in the formation of a tumor, and the production of nutrients that can be used by *Agrobacterium*.

The T-strand region of the Ti plasmid is delimited by the two border sequences: the Left and Right Border (LB and RB). Any DNA sequence present between the LB and RB will be transferred to a plant cell. This allows for the generation of transgenic plants in which DNA sequences of choice have been integrated. In the laboratory, the natural host range of *Agrobacterium* has been expanded to include, amongst others, monocotyledonous plants and fungi, making *Agrobacterium* an invaluable tool for biotechnology.

The research described in this thesis was aimed at enlarging the understanding of the *Agrobacterium* protein VirD2. Using its relaxase domain to nick the border sequences delimiting the T-region, VirD2 is responsible for processing of the T-strand, to which it remains covalently attached. In addition, the translocation of the T-strand into the recipient cell is directed by VirD2. We aimed to elucidate the requirements of different domains of VirD2 for translocation. Furthermore, we also developed VirD2 into a tool for site-directed mutagenesis in plants. We used the model plant *Arabidopsis thaliana* (thale cress), since rapid transformation techniques are available and the plant has a relatively short life cycle.

Chapter 1 describes in detail the mechanism of *Agrobacterium*-mediated transformation (AMT) and its biotechnological applications. Mechanistically, AMT is related to plasmid conjugation and pathogenesis of certain bacteria in that the translocation of DNA and proteins occurs via a Type 4 Secretion System (T4SS). The T4SS is a large protein structure, adapted to translocate protein (and protein-bound DNA) to recipient cells. T-strands preferentially integrate into double-strand breaks (DSBs) in the genome of the recipient cell. To artificially induce DSBs in eukaryotic cells, two classes of proteins are commonly used: the zinc finger nucleases and the homing endonucleases, both with advantages and drawbacks.

The research described in **Chapter 2** was aimed at defining the essential domains of VirD2. To this end, we created VirD2 truncations, consisting of the relaxase domain – responsible of the processing of the T-strand – while a large domain of unknown function (DUF) was not at all or only partially present. For its translocation to plant cells, a translocation signal was required at the C-terminal end of VirD2. When appropriate, we replaced it by the translocation signal of the unrelated protein VirF. In that manner, we found that DUF can be deleted completely when the translocation signal of VirF is present. When the endogenous C-terminal sequences of VirD2 are present, only 60 amino acid residues of DUF (DUF-60) are required for translocation. The fact that the translocation signal of an unrelated protein can restore T-strand transformation provides further evidence that T-strand translocation is protein-driven, rather than driven by the T-strand, as suggested by earlier models regarding the function of the T4SS of *Agrobacterium*. In this study, we have provided compelling evidence supporting a change of opinion regarding the T4SS function.

In **Chapter 3**, the role of DUF-60 is further investigated. The results of Chapter 2 could be explained by assuming DUF-60 is required for an interaction, either with a factor within plants or a factor within *Agrobacterium*. The C-terminal end of VirF can apparently substitute for this function, while the C-terminal end of VirD2 alone is insufficient and required support from DUF-60. We investigated this by inoculating *Agrobacterium* strains expressing relevant VirD2 mutants onto two different plant species, *Nicotiana glauca* and *Kalanchoë tubiflora*. These experiments showed that DUF-60 does not fulfill a role in these plant species. Instead, using GFP-tagged VirD2 mutants, we discovered that DUF fulfills an important function with *Agrobacterium* cells. We found that DUF is of crucial importance for the localization of VirD2 to the cell poles of *Agrobacterium*, and polar localization of VirD2 via DUF-60 therefore seems essential for the virulence of an *Agrobacterium* strain. Remarkably, the C-terminal end of VirF can restore polar localization of VirD2-204, and can therefore substitute for DUF-60.

It is known that induction of DSBs in the genome induces DNA repair via homologous recombination, which is the desired pathway for the correct integration of foreign DNA, such as the T-strand. The technology developed thus far employs zinc finger nucleases (ZFNs) or homing endonucleases (HEs) for induction of site-specific DSBs in complex genomes. VirD2 is potentially a very interesting candidate protein for mediating site-directed mutagenesis in plants. It has the unique advantage that it is covalently bound to the T-strand, which can be used as repair template for the incorporation of desired mutations into the plant genome. In **Chapter 4** experiments concerning novel types of

DSB-inducing fusion proteins based on VirD2 are described. Fusions were made consisting of the VirD2 protein, or a large part thereof, combined with 1 up to 6 zinc finger (ZF) domains, as well as complete ZFNs. Furthermore, we made fusions of VirD2 and two different homing endonucleases, I-SceI and HO. To ensure passage through the T4SS, we added the translocation signal of VirF (F). It was then tested if these proteins could still be translocated from *Agrobacterium* to *Arabidopsis* root cells. Remarkably, all fusion proteins could be translocated. However, the translocation efficiency varied widely between the different fusion proteins. An increasing number of ZFs caused a steep decline in translocation efficiency of VirD2-based fusion proteins. While fusion of VirD2 to HO also severely decreased translocation efficiency, similar types of fusions to I-SceI still translocated at high efficiency and were shown to display I-SceI nuclease activity when expressed *in planta*.

In **Chapter 5** we determined that the VirD2-I-SceI-F and VirD2-204-I-SceI-F fusion proteins remained active after AMT. Since passage through the T4SS may involve (partial) unfolding of the protein, this was not self-evident. Activity was determined by screening for damaged I-SceI recognition sites in a plant line in which a targeting locus was pre-inserted. In 3 out of 950 plants, a damaged I-SceI recognition site was discovered. For the development of strategies for gene targeting, the process in which a particular gene is altered without disturbing the rest of the genome, the VirD2-I-SceI-F fusion protein holds promise. This is because the VirD2-I-SceI fusion protein is administered transiently and is covalently bound to the T-strand, ensuring the T-strand is in the vicinity of the DSB when it is made. Also, current advances in modification of HE recognition specificity will result in the creation of HEs suitable to create DSBs in endogenous loci.

In conclusion, the data presented in this thesis reveal previously unknown functions of VirD2. The requirements of VirD2 domains for T-complex translocation have been elucidated and a function has been assigned to the previously uncharacterized DUF domain. Following up on these results, we have created novel recombinant effector proteins that combine the T-strand processing and translocation properties of VirD2 and the (potential) DSB-inducing activity of a foreign protein. The VirD2-I-SceI-F fusion protein turned out to be translocated at a high efficiency, and showed DSB-inducing activity *in planta* after *Agrobacterium*-mediated transformation. These results show that VirD2 is a promising candidate for further development into a tool for the induction of transient effects in organisms susceptible to *Agrobacterium*-mediated transformation.